学位論文（博士）

Doctoral dissertation

Hesperetin inhibits sphingosylphosphorylcholine-induced vascular smooth muscle contraction by regulating the Fyn/Rho-kinase pathway
（ヘスペレチンは、Fyn / Rho キナーゼ経路を調節することにより、スフィンゴシルホスホリルコリン誘発性の血管平滑筋収縮を阻害する）

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Abstract

Cardiovascular diseases are the leading cause of mortality and disability worldwide. We have previously found that sphingosylphosphorylcholine (SPC) is the key molecule leading to vasospasm. We have also identified the SPC/Src family protein tyrosine kinase Fyn/Rho-kinase (ROK) pathway as a novel signaling pathway for Ca^{2+}-sensitization of vascular smooth muscle (VSM) contraction. The present study aimed to investigate whether hesperetin can inhibit the SPC-induced contraction with little effect on 40 mM K^+-induced Ca^{2+}-dependent contraction and to elucidate the underlying mechanisms. Hesperetin significantly inhibited the SPC-induced contraction of porcine coronary artery smooth muscle strips with little effect on 40 mM K^+-induced contraction. Hesperetin blocked the SPC-induced translocation of Fyn and ROK from the cytosol to the membrane in human coronary artery smooth muscle cells (HCASMCs). SPC decreased the phosphorylation level of Fyn at Y531 in both VSMs and HCASMCs and increased the phosphorylation levels of Fyn at Y420, myosin phosphatase target subunit 1 (MYPT1) at T853 and myosin light chain (MLC) at S19 in both VSMs and HCASMCs, which were significantly suppressed by hesperetin. Our results indicate that hesperetin inhibits the SPC-induced contraction at least in part by suppressing the Fyn/ROK pathway, suggesting that hesperetin can be a novel drug to prevent and treat vasospasm.

Background

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide. More than four in five CVD deaths, such as those due to heart attacks and strokes, result from the abnormal contraction of vascular smooth muscle (vasospasm). Various signal transduction pathways regulate the contraction and relaxation of vascular smooth muscle (VSM).[1] Previous studies have revealed regulatory processes of VSM contraction, such as G-protein-coupled inhibition of myosin light chain phosphatase (MLCP), the regulation of myosin light chain kinase (MLCK) by other kinases, and the functional effects of smooth muscle myosin isoforms.[2] Abnormalities of these regulatory mechanisms and isoform variations may contribute to vasospasm, leading to CVDs.[2, 3]

We previously identified for the first time that Rho-kinase (ROK)-mediated Ca^{2+}-sensitization of smooth muscle directly induces smooth muscle contraction by phosphorylating myosin light chain (MLC).[4] ROK-mediated Ca^{2+}-sensitization of smooth muscle plays a pivotal role in the abnormal contraction of VSM. Although G-protein regulates the Ca^{2+}-sensitization of smooth muscle,[5] sphingosylphosphorylcholine (SPC) induces ROK-mediated Ca^{2+}-sensitization independently of G-protein. We have demonstrated that SPC is a novel messenger for ROK-mediated Ca^{2+}-sensitization of VSM contraction, which plays a critical role in the pathogenesis of vasospasm.[6, 7] Nakao *et al.* demonstrated that the SPC-induced Ca^{2+}-sensitization of VSM contraction in porcine coronary arteries is mediated specifically by Src family protein tyrosine kinases (SFKs) and revealed that SPC induces the

translocation of Fyn, a member of the SFKs, from the cytosol to the cell membrane in primary cultured rat vascular smooth muscle cells (VSMCs). The translocation and activation of Fyn play critical roles in the Ca^{2+}-sensitization of VSM contractions mediated by the SPC/ROK pathway.[8] We previously found that eicosapentaenoic acid (EPA) [8] selectively inhibits the SPC-induced Ca^{2+}-sensitization of VSM contractions *in vitro* by suppressing the translocation of Fyn from the cytosol to the cell membrane. Clinical studies have shown that EPA significantly reduces the occurrence of cerebral vasospasm following subarachnoid hemorrhage.[9] Thus, the SPC/Fyn/ROK pathway plays a vital role in human vasospasm. EPA is obtained mainly from fish oil. However, pollution and excessive utilization of marine resources have restricted the procurement of EPA. Therefore, identifying highly effective and easily accessible natural chemical substances to prevent and treat the SPC-induced abnormal VSM contraction is crucial to prevent and treat CVDs. In this regard, natural bioactive ingredients from plants and fruits have become potential alternatives.

Hesperetin, a natural flavonoid, has a wide spectrum of pharmacological effects, such as anti-inflammatory,[10] antioxidant,[11] antitumor,[12] neuroprotective effects,[13] and antiangiogenic effects.[14] Although other studies have shown that hesperetin induces vasorelaxation,[15, 16] knowledge of the underlying mechanism is not comprehensive. Additionally, the effect of hesperetin on the SPC-induced vasospasm remains unclear. In the present study, we investigated the inhibitory effects of hesperetin on the SPC-induced VSM contraction in porcine coronary arteries and human coronary artery smooth muscle cells (HCASMCs) and explored the potential mechanism involved in

suppressing the SPC/Fyn/ROK pathway. Our research provided new ideas to prevent and treat cardiovascular diseases.

Aim

The present study aimed to investigate the effects of hesperetin on the SPC-induced VSM contraction and to explore the potential mechanism by which hesperetin affects the SPC-induced abnormal VSM contraction in porcine coronary arteries and human coronary artery smooth muscle cells (HCASMCs), providing new ideas to prevent and treat cardiovascular diseases.

Methods

Subjects

The protocols for this research project have been approved by the Institutional Ethics Committee of Yamaguchi University and were conducted in conformity with institutional guidelines. The porcine (age: 5 ~ 6 months; weight: approximately 120 kg; the ratio of male to female porcine was 4:6) coronary arteries (20 to 30 mm from the origin of the proximal portion of left anterior descending arteries) tissues were obtained from a local public abattoir (Kitakyushu Municipal Meat Inspection and Control Center, Japan). Human (male) coronary artery smooth muscle cells (HCASMCs) were purchased from Kurabo Industries, Ltd. (Osaka, Japan).

Methods

Materials and reagents

Hesperetin (purity ≥ 96%) was purchased from Wako Pure Chemical (Osaka, Japan) and dissolved in 100% DMSO to make a 100 mM stock solution. The stock solution was stored at -20°C and then diluted to the final concentrations before use. SPC was purchased from Enzo Life Sciences Inc. (Enzo Biochem, Inc., New York, USA). Bradykinin (BK) was purchased from Peptide Institute, Inc. (Osaka, Japan).

Tissue preparation

The porcine coronary arteries tissue specimens were kept in ice-cold Krebs solution (123 mM NaCl, 4.7 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, and 11.5 mM D-glucose) for transportation to the laboratory. The Krebs solution was gassed with a mixture of 95% O_2 and 5% CO_2 for at least 15 min.

VSM tissues without the endothelium and adventitia were cut into strips (0.7 mm × 4 mm). Complete removal of the endothelium from the strips was confirmed by a lack of relaxation in response to 1 μM BK. All the procedures were subject to approval by the Institutional Animal Care and Use Committee of Yamaguchi University and were conducted according to institutional guidelines.

Measurement of force in VSM

Isometric contractions of the coronary artery smooth muscle strips without the endothelium and adventitia were measured using a force transducer (TB-611T, Nihon-Kohden, Tokyo, Japan), as described previously.[17] The experimental process is briefly summarized as follows: These strips were mounted vertically in an organ bath filled with Krebs solution, gassed with 5% CO_2/95% O_2, and maintained at 37°C in a force transducer. After the relaxed smooth muscle tissue strips were stable in the solution for 15 minutes, we stimulated smooth muscle strips with 118 mM K^+ for 5 minutes. Next, 118 mM K^+ solution was washed out with Krebs solution. After 5 minutes, we applied resting tension. After five minutes of continuous resting tension, 118 mM K^+ was used to induce depolarization contraction of smooth muscle strips for an additional five minutes. Next, the above cycle process was repeated until the depolarization-induced contraction caused by 118 mM K^+ reached a maximum, i.e., the resting tension was optimized. After the resting tension was optimized, the effects of post- and pre-treatment hesperetin on the maximum and steady-state forces of contractions induced by 30 μM SPC or 40 mM K^+ were examined. The extent of contraction inhibition by hesperetin is described as the percentage by which the contraction induced by 30 μM

SPC or 40 mM K^+ was inhibited.

Cell culture

Human (male) coronary artery smooth muscle cells (HCASMCs, purchased from Kurabo, Osaka, Japan) were cultured in HuMedia SG2 (Kurabo, Osaka, Japan) containing 5% fetal bovine serum (FBS), 0.5 ng/ml of human epidermal growth factor, 2 ng/ml of human fibroblast growth factor-B, 5 μg/ml of insulin, 50 μg/ml of gentamycin, and 50 ng/ml of amphotericin B. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. HCASMCs were used for experiments within 3-10 passages after culture initiation. The cells were subjected to serum starvation for 24 h using HuMedia SB2 (Kurabo, Osaka, Japan) before stimulation with or without SPC or hesperetin.

Time-lapse recording of HCASMC contraction

HCASMCs were cultured in 35-mm dishes (BD Falcon, New York, USA) at a density of 1×10^5 cells/dish. When the cell confluence reached 90-100%, the medium was replaced with FBS- and growth factor-free HuMedia SB2 to obtain the hypercontractile type of HCASMCs. After treatment with HuMedia SB2 for 24 h, the cells were pretreated with hesperetin for 30 min at 37°C. Next, 30 μM SPC was added to the medium, and time-lapse recording of HCASMC contraction was performed using an all-in-one fluorescence microscope BZ-9000 (Keyence, Osaka, Japan).

Immunofluorescence staining

Immunofluorescence staining was performed as described previously.[18] Briefly, HCASMCs were seeded on sterile coverslips that had previously been coated with 200

μL of 0.3% gelatin at room temperature for 30 min. After the cell confluence reached 90-100%, the cells were preincubated with or without hesperetin for 30 min and then treated with SPC for 10 min. Next, the HCASMCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS for 2 min, blocked in NanoBio blocker solution (Nano Bio Tech Co., Ltd, NY, USA) and diluted in PBS for 60 min at room temperature. After that, the HCASMCs were stained with primary anti-ROCK (dilution time: 1:100; sc-1851, Santa Cruz, Dallas, TX, USA) and anti-Fyn (dilution time: 1:100; 610164, BD Biosciences, NY, USA) antibodies at 4°C overnight, followed by staining with Alexa Fluor 488-conjugated donkey anti-goat IgG (A32814; Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 546-conjugated goat anti-mouse IgG (A11003; Thermo Fisher Scientific, Waltham, MA, USA) secondary antibodies. Before observation, the cells were mounted with PermaFluor aqueous mounting medium (Thermo Fisher Scientific, Waltham, MA, USA). The field of view was randomly selected to observe stained cells under an all-in-one fluorescence microscope BZ-9000 (Keyence, Osaka, Japan). Fluorescence intensity profile analysis was performed using ImageJ software.

Western blot analysis

Tissue and cell proteins were prepared as described previously.[17] Briefly, VSM tissues were preincubated with hesperetin (30 μM) in Krebs solution at 37°C for 30 min and then were stimulated with SPC (30 μM) in the absence (vehicle) or presence of hesperetin in Krebs solution. The tissues were rapidly treated, frozen and then fractured using SK mill (SK-100; Tokken, Japan). The tissue samples were lysed in RIPA buffer

(Wako, Osaka, Japan). HCASMCs were stimulated with SPC (30 μM) after pretreatment with hesperetin (30 μM for 30 min) or vehicle and then were collected in RIPA buffer. The supernatants were kept in sodium dodecyl sulfate (SDS) loading buffer at 95°C for 5 min for western blot analysis after centrifugation at 10,000 × *g* and 4°C for 5 min.

The tissue and cell protein lysates were separated by 10% or 12.5% SDS-PAGE and subjected to immunoblotting with appropriate antibodies against Fyn (1:1,000; ab125016; Abcam, Cambridge, MA, USA), anti-Fyn (1:1000; 610164; BD Biosciences, NY, USA), myosin phosphatase target subunit 1 (MYPT1) (H-130) (1:500; sc-25618; Santa Cruz, Dallas, TX, USA) , p-MYPT1 (T853) (1:500; sc-17432; Santa Cruz, Dallas, TX, USA), myosin light chain (MLC) (1:1,000; M4401; Sigma-Aldrich, St. Louis, MO, USA), p-MLC (S19) (1:1,000; 3671S; Cell Signaling, Danvers, MA, USA) and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (1:5,000; MAB374; Chemicon, Waltham, MA, USA). All the primary antibodies were diluted with 5% nonfat milk in Tris-buffered saline-0.05% Tween-20 (TBS-T). HRP-conjugated anti-mouse IgG (W4021) and anti-rabbit IgG (W4011) secondary antibodies (1:2,000; Promega, Madison, WI, USA) were diluted with TBS-T. GAPDH was used as a loading control. The signals were visualized using SuperSignal West Pico chemiluminescent substrates (Thermo Fisher Scientific, Waltham, MA, USA) and evaluated using Quantity One software with ChemiDoc XRS-J (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Immunoprecipitation and estimation of Fyn activation

We performed Fyn immunoprecipitation in porcine coronary arterial smooth muscle

tissues and HCASMCs. Briefly, tissue and cell lysates were prepared as described previously[17] and clarified by centrifugation at 10,000 × *g* and 4 ℃ for 10 min. Total protein (100 μg) was removed for immunoprecipitation after the protein concentration was detected. First, suspended Protein A/G Plus Agarose (Santa Cruz, Dallas, TX, USA) was placed into 1.5 mL tubes and washed three times with RIPA buffer. Second, normal mouse IgG (Santa Cruz, Dallas, TX, USA) was used to remove nonspecific proteins. One microgram of anti-Fyn antibody (610164; BD Biosciences, New York, USA), 20 μL of resuspended protein A/G agarose, and the supernatant without nonspecific proteins were mixed thoroughly and rotated overnight at 4 ℃. Next, the sediment was collected via centrifugation at 1,000 ×*g* and 4 ℃ for 5 min. The sediment was washed four times using RIPA buffer, the supernatant was removed, and 40 μL of sodium dodecyl sulfate (SDS) sample buffer was added. The samples were incubated at 95 ℃ for 5 min, and the supernatant was subjected to western blot analysis after centrifugation at 10,000 ×*g* and 4 ℃ for 5 min. We analyzed Fyn activation via western blot analysis using a phospho-Src family (Y416; corresponding to Y420 in human and pig Fyn) polyclonal antibody (1:1,000; 2101, Cell Signaling, Danvers, MA, USA) and a phospho-Src family (Y527; corresponding to Y531 in human and pig Fyn) polyclonal antibody (1:1,000; 2105S; Cell Signaling, Danvers, MA, USA).

Transfection of pcDNA6 myc/His A FynYF

pcDNA6 myc/His A FynYF was transfected as described previously.[19] Briefly, human cDNAs encoding the constitutively active form of Fyn with the Y530F mutation (ca-Fyn) were subcloned into the pcDNA6/myc-His A vector (Invitrogen, Thermo Fisher

Scientific, Waltham, MA, USA). The construct was verified by DNA sequencing. HCASMCs were trypsinized, counted and divided into at least 5×10^5 cells per tube when the cell confluence reached 90-100%. An Amaxa Human AoSMC Nucleofector Kit (Lonza, Tokyo, Japan) was used for the nucleofection of pcDNA6 myc/His A FynYF. HCASMCs were transfected with 2 μg of pcDNA6/myc-His A or pcDNA6 myc/His A FynYF using a Nucleofector II device following the manufacturer's instructions for the kit. PmaxGFP from the kit was used to monitor the transfection efficiency (> 80%). HCASMCs were serum starved for 24 hours after transfection for 48 h. Next, the cells were treated with 30 μM hesperetin for 30 min. Immunoprecipitation and western blot were applied to analyze the activation of Fyn and ROK in HCASMCs.

Analysis

All the experimental data were presented as means ± standard deviation, and each experiment was performed at least three times. The data were analyzed using Prism 8.4 (GraphPad Prism software, San Diego, California, USA), and significant differences were determined by one-way ANOVA followed by the Student-Newman-Keuls post hoc test with 95% confidence and by unpaired t-test. A difference with a level of $P < 0.05$ was accepted as statistically significant.

Results

Effects of post- and pretreatment hesperetin on SPC- and 40 mM K^+ depolarization-induced contractions of VSM

To detect the effects of hesperetin on the SPC- and 40 mM K^+-mediated depolarization-induced contractions of VSM, we observed the direct effects of hesperetin on porcine coronary artery VSM strips. Hesperetin exerted different effects on VSM contraction induced by the two stimuli (Fig. 1). The typical inhibitory effects of hesperetin at 30 μM on 40 mM K^+ depolarization- and SPC-induced contractions of VSM are displayed in Fig. 1A and Fig. 1C, respectively. At a concentration of 30 μM, hesperetin strongly inhibited SPC-induced Ca^{2+}-sensitization of VSM contraction, resulting in a level 79.4 ± 7.4% lower than that in the vehicle control group (Fig. 1B). However, hesperetin minimally affected 40 mM K^+ depolarization-induced contraction, reducing it by only 1.95 ± 2.34%. Additionally, the inhibitory effects of hesperetin on SPC-induced contraction were concentration-dependent (Fig. 1D). The IC50 values for hesperetin inhibition of SPC- and KCl-induced contraction were 13.94 μM and 65.17 μM, respectively. We also observed that the inhibitory effect of hesperetin was reversible when we changed the media to Krebs solution to wash out the hesperetin after we observed a direct inhibitory effect of hesperetin on SPC-induced contraction (Fig. 1I).

To investigate the protective effect of hesperetin against the abnormal VSM contraction induced by SPC, we also detected the effects of hesperetin pretreatment on VSM contractions induced by 40 mM K^+ depolarization and SPC in porcine coronary artery VSM strips. Pretreatment with hesperetin had little effect on the 40 mM K^+

depolarization-induced contraction of VSM, reducing it by only 14.6 ±3.6% (Fig. 1E, H). After the VSM strips were preincubated with 30 μM hesperetin for 30 min at 37°C in Krebs solution, SPC was added to Krebs solution to stimulate abnormal VSM contraction. SPC stimulation caused minor contractions in VSM strips pretreated with hesperetin (Fig. 1G) but caused strong contractions in VSM strips pretreated with the vehicle control (Fig. 1F). The findings showed that pretreatment with hesperetin significantly inhibited the SPC-induced contraction by 80.3 ±6.6% (Fig. 1H).

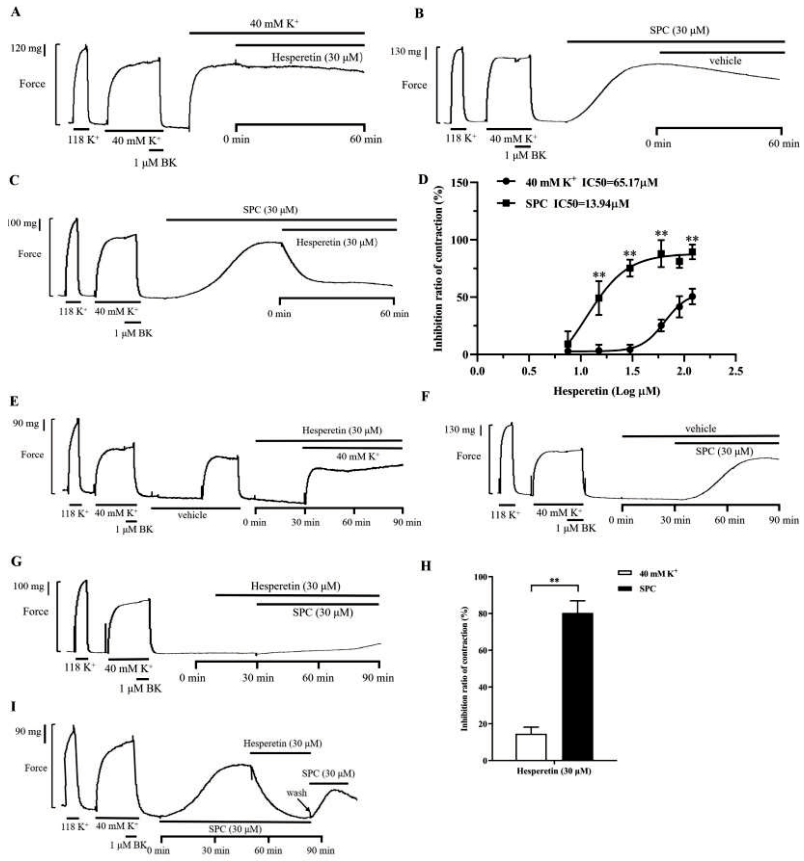

Figure 1. The effects of hesperetin (post- and pre-treatment) on the SPC and 40 mM K^+ depolarization-induced contractions of VSM and the reversible effect of hesperetin.

(A) and (C) Representative recordings showed the direct effects of hesperetin (30 μM) on the SPC- and 40 mM K^+ depolarization-induced contractions in porcine coronary arteries strips. (B) Representative recordings showed the effect of the vehicle on the SPC-induced contraction in

porcine coronary arteries strips. (D) The inhibitory ratios of hesperetin on the SPC- and 40 mM K^+ depolarization-induced contractions of VSM. (E) Representative recordings showing the effect of pretreated hesperetin on 40 mM K^+ depolarization-induced contraction of VSM after vehicle pretreatment. (F, G) Representative recordings showing the effects of pretreated vehicle and hesperetin on the SPC-induced contraction of VSM. (H) The inhibitory ratios of pretreated hesperetin (30 μM) on the SPC- and 40 mM K^+ depolarization-induced contraction of VSM. (I) Representative recordings showing the reversible effect of hesperetin on the SPC-induced contraction of VSM. Data were presented as the mean ± standard. n = 3 ~ 6. $^{**}P < 0.01$.

Effects of preincubation with hesperetin on HCASMC contraction induced by SPC

To clarify the mechanism by which hesperetin inhibited the SPC-induced VSM contraction, HCASMCs were further evaluated. Because SPC induced stable and time-dependent contraction in HCASMCs,[17] a finding that is consistent with its effects on porcine coronary artery VSM strips, we observed the effects of hesperetin on the SPC-induced contraction in HCASMCs. The SPC-induced contraction of HCASMCs resulted in a cell morphological change from spindle-shaped to round (see Supplementary Video 1). SPC induced prominent and time-dependent contraction in HCASMCs (Fig. 2). By contrast, after preincubating HCASMCs with hesperetin for 30 min, SPC induced little contraction and caused little morphology change (see Supplementary Video 2).

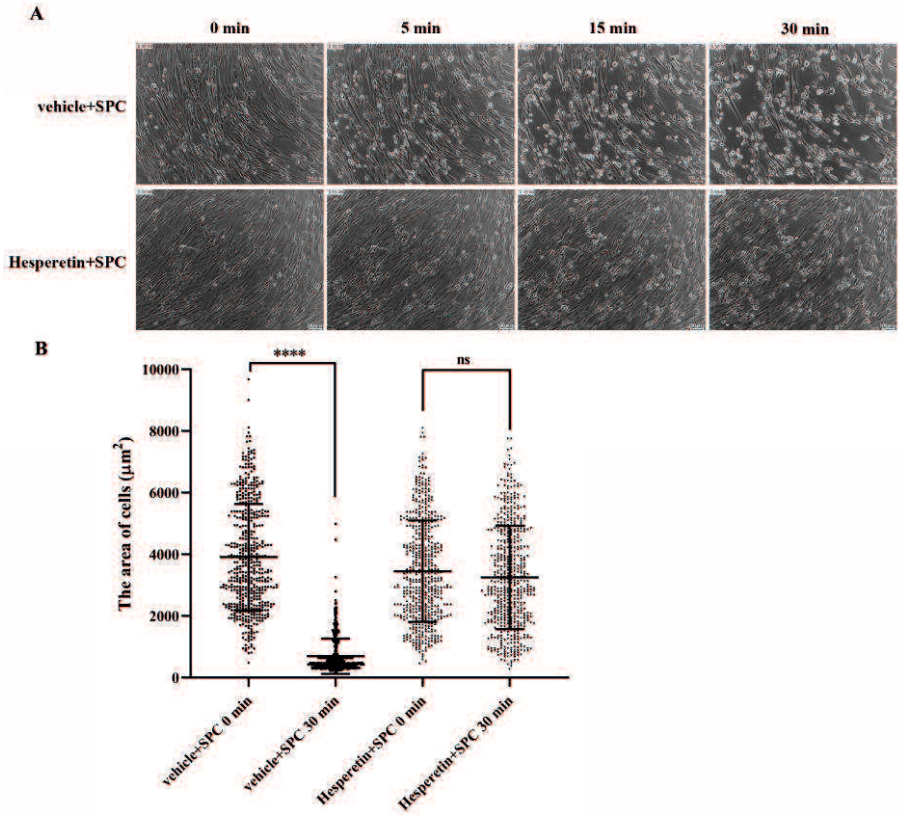

Figure 2. Effects of hesperetin on the SPC-induced contraction in HCASMCs. (A) The time lapse observation was carried by the microscope, while the selected images showed the differences between the two groups at different times. Scale bar = 100 μm. (B) The quantified change of cell contraction after SPC treatment in vehicle group and hesperetin pre-treated group was displayed in statistical analysis. ****$P < 0.0001$; ns means no statistically significant.

Hesperetin inhibits Fyn and ROK translocation induced by SPC in HCASMCs

The SPC/Fyn/ROK pathway plays a vital role in VSM contraction.[6] We observed that hesperetin inhibited the SPC-induced contraction and hypothesized that its mechanism of action involves inhibiting the SPC/Fyn/ROK pathway. Immunofluorescence staining was performed to observe the effect of hesperetin on the SPC-induced translocation of Fyn and ROK from the cytosol to the cell membrane in HCASMCs. Changes in the membrane/cytosol (M/C) signaling intensity ratios of Fyn and ROK were statistically

analyzed. SPC induced the translocation of Fyn and ROK from the cytosol to the membrane, as measured by immunofluorescence staining (Fig. 3A), markedly increasing the M/C ratio (Fig. 3B). By contrast, SPC did not induce Fyn or ROK translocation in HCASMCs preincubated with hesperetin, indicating that hesperetin inhibits the SPC-induced translocation of Fyn and ROK from the cytosol to the membrane.

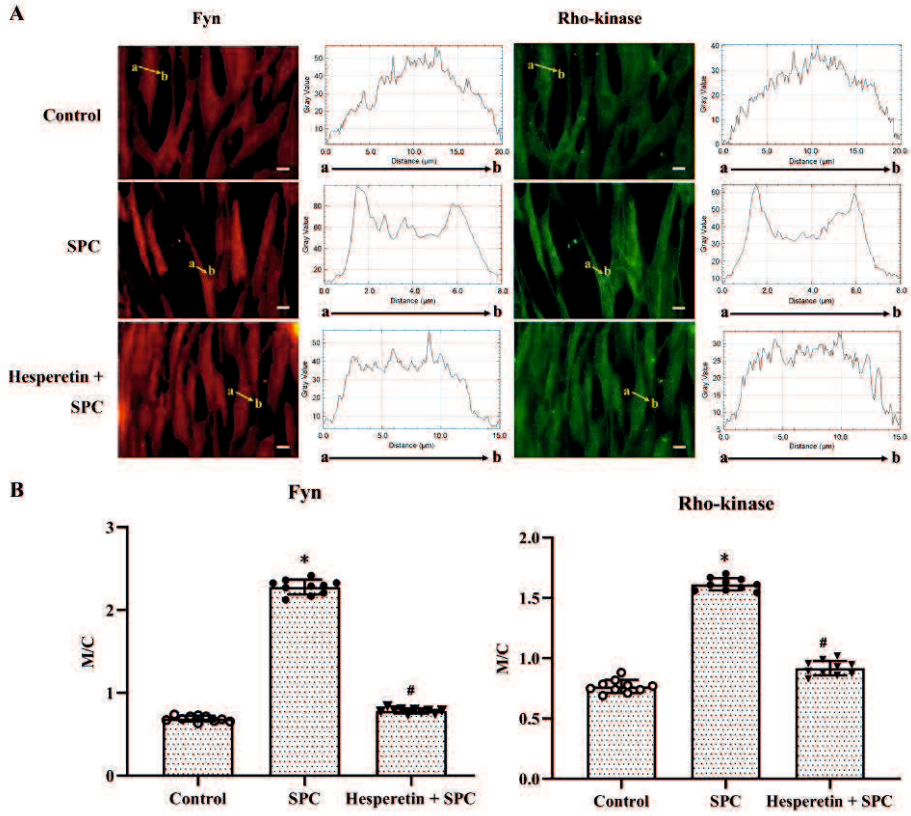

Figure 3. Hesperetin inhibits the SPC-induced translocations of Fyn and ROK in HCASMCs.

(A) Cells were fixed with paraformaldehyde and stained with anti-Fyn and anti-ROK antibody. Fyn was labeled with red, while ROK was labeled with green. The curve on the right side of immunofluorescence represents the changes of intensity along with "a" to "b" arrow. The plot profile of the intensity from "a" to "b" arrow was carried by Image J software. Scale bar =10 μm. (B) Statistical analysis of the ratios of Fyn and ROK fluorescence intensities on membrane (M) to

that on cytosol (C), M/C. Data are the means of four experiments in which at least 10 cells were analysed per experiment. *$P < 0.05$ versus control group; #$P < 0.05$ versus SPC-treated group.

Hesperetin inhibits the SPC-induced Fyn and ROK activation in both VSM tissues and HCASMCs

Although hesperetin inhibits the SPC-induced translocation of Fyn and ROK, whether hesperetin inhibits the SPC-induced activation of two kinases remains unclear. To further verify whether hesperetin inhibited VSM contraction by preventing SPC from activating Fyn and ROK, we measured Fyn activation by detecting tyrosine phosphorylation at 420 and 531 sites of the SH1 kinase domain of Fyn after immunoprecipitation. We also analyzed threonine phosphorylation at site 853 of MYPT1 (p-MYPT1 T853) as a readout of ROK activity in VSM and HCASMCs.[20] The immunoblotting results showed that SPC increased p-Src (Y420) and decreased p-Src (Y531) of Fyn, an effect that was significantly alleviated by hesperetin in both VSM (Fig. 4A, B) and HCASMCs (Fig. 4C, D) ($P < 0.05$), suggesting that hesperetin inhibits the SPC-induced Fyn activation in both VSM and HCASMCs. Simultaneously, although SPC significantly increased p-MYPT1 (T853), this effect was abolished when VSM tissues and HCASMCs were preincubated with hesperetin, indicating that hesperetin inhibits the SPC-induced ROK activation in both VSMs and HCASMCs (Fig. 4E, F, G, H).

To further confirm the inhibitory effect of hesperetin on the activation of Fyn and ROK, we transfected constitutively active Fyn (ca-Fyn) into HCASMCs following hesperetin treatment (Fig. 4I, J). Compared with the Fyn activity in the mock vector groups, ca-

Fyn increased Fyn and ROK activities by 3.84 ± 0.30-fold and 2.72 ± 0.32-fold, respectively. By contrast, ca-Fyn with hesperetin treatment showed much lower activities of Fyn (1.85 ± 0.35-fold) and ROK (1.26 ± 0.38-fold) than ca-Fyn did, indicating that hesperetin inhibits Fyn and ROK activations in HCASMCs.

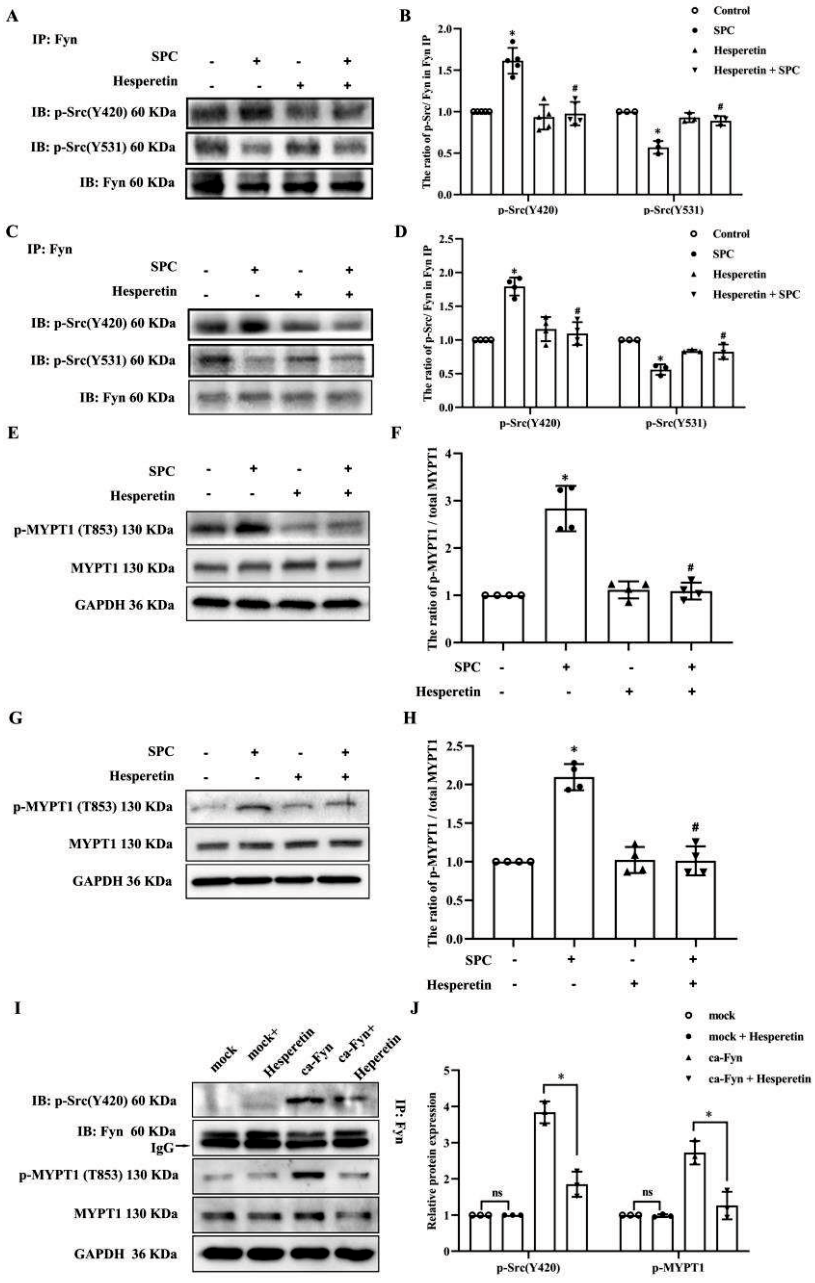

Figure 4. The effects of hesperetin on the SPC-induced Fyn and ROK activation in VSM tissues and HCASMCs. (A and C) Analysis of Fyn activation via phospho-Src immunoblotting

after Fyn immunoprecipitation from VSM (A) and HCASMC (C) samples. Representative western blot showed the variation of p-Src (Y420) and p-Src (Y531) in different groups. Total Fyn (antibody: ab125016; Abcam, Cambridge, MA, USA) was used as control. (E and G) Phosphorylation of MYPT1 (T853) in VSMs (E) and HCASMCs (G) were analysed via western blot. (B, D, F and H) Statistical analysis revealed the change of Fyn activation (proportion of Fyn phosphorylation at Y420 and Y531 to total Fyn after Fyn immunoprecipitation) (B, D) and ROK activation (proportion of MYPT1 phosphorylation at site Thr853 to total MYPT1) (F, H) in both VSMs (B, F) and HCASMCs (D, H). Data were presented as the mean ± SD. n = 3 ~ 5. *$P < 0.05$ versus control group; # $P < 0.05$ versus SPC-treated group. (I) HCASMCs were transfected with ca-Fyn or mock for 48 h and serum-starved for 24 h, followed by hesperetin treatment for 30 min. Those cells were lysed and analysed to the expression p-Src (Y420) after Fyn immunoprecipitation and p-MYPT1 (T853). Representative western blot showed the variation of p-Src (Y420) and p-MYPT1 (T853) in mock or ca-Fyn groups with or without hesperetin treatment. Total Fyn (antibody: 610164; BD Biosciences, NY, USA) and total MYPT-1 were used as control, respectively. (J) Statistical analysis revealed the change of Fyn activation (proportion of Fyn phosphorylation at Y420 to total Fyn after Fyn immunoprecipitation) and ROK activation (proportion of MYPT1 phosphorylation at site Thr853 to total MYPT1) in HCASMCs. Data were presented as the mean ± SD, n = 3. *$P < 0.05$; ns means no statistically significant.

Hesperetin inhibits MLC phosphorylation induced by SPC in both VSM tissues and HCASMCs

Phosphorylation of MYPT1 (T853) inhibits myosin phosphatase, increasing the phosphorylation of MLC (p-MLC) at S19 and leading to vasoconstriction.[21] Western

blot analysis was performed to observe the effect of hesperetin on the p-MLC (S19) induced by SPC in VSM tissues and HCASMCs. SPC significantly enhanced p-MLC(S19) (2.05 ± 0.24-fold), and this effect was blocked by hesperetin (1.15 ± 0.08-fold) in VSM tissues (Fig. 5A, B). Additionally, SPC-induced p-MLC (S19) was significantly lower in HCASMCs pretreated with hesperetin (1.36 ± 0.03-fold) than in vehicle control-treated cells (2.91 ± 0.95-fold; Fig. 5C, D).

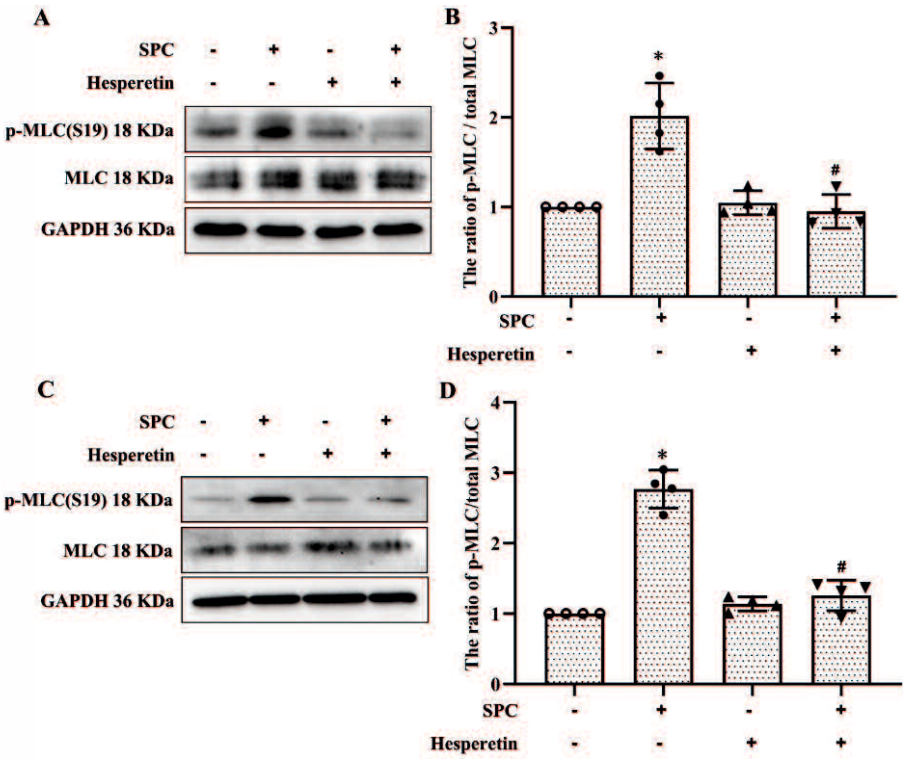

Figure 5. The effects of hesperetin on the SPC-induced myosin light chain (MLC) phosphorylation in VSM tissues and HCASMCs. (A and C) Representative western blot showed the variation of p-MLC from different groups in porcine coronary artery VSM strips (A) and HCASMCs (C). (B and D) Statistical analysis reveal that the increased MLC phosphorylation (proportion of MLC phosphorylation at site Ser19 to total MLC) induced by SPC were inhibited by hesperetin in both VSMs (B) and HCASMCs (D). Data were presented as the mean ± SD. n = 4. * $P < 0.05$ versus control group; # $P < 0.05$ versus SPC-treated group.

Discussion

Hesperetin, a flavonoid compound from citrus fruits, is a candidate therapeutic agent that may benefit the cardiovascular system.[22] Various studies have illustrated the vasodilatory effect of hesperetin from different perspectives. Orallo *et al.* believed that the vasorelaxant effects of hesperetin are due to the inhibition of PDE1 and PDE4 activities.[16] Liu and colleagues demonstrated that hesperetin has an inhibitory effect on the vasocontraction induced by depolarization, U46619 and Ca^{2+} influx.[23] Additionally, hesperetin has been proven to promote the nitric oxide production by endothelial cells.[24,25,26] However, the effect of hesperetin on the SPC-induced abnormal contraction has remained unclear. Here, we present, for the first time, evidence that hesperetin can inhibit the SPC-induced abnormal contraction by suppressing the translocation and activation of Fyn and ROK in VSM. Our findings suggest that hesperetin is beneficial to treat and prevent vasospasm, proposing a novel inhibitory mechanism of hesperetin on vascular smooth muscle contraction.

SPC is a sphingomyelin metabolite that occurs naturally in plasma, the levels of which are low (50 nM in plasma, 130 nM in serum) under normal physiological conditions.[27] Additionally, SPC plays a physiological role in regulating the heart.[27, 28] However, abnormal contraction of VSM induced by higher concentrations of SPC-mediated Ca^{2+}-sensitization has been proposed as a major cause of vasospasm diseases,[29, 30] indicating that SPC is considered a spasmogen. Additionally, we have previously demonstrated that SPC induced Ca^{2+}-sensitization of VSM contraction in bovine, porcine, and human arterial strips.[6, 7, 31] The *in vitro* model experiment in our

present study simulated the exposure of blood vessels to high concentrations of SPC *in vivo* to observe the inhibitory effect of hesperetin on the vasospasm induced by SPC. Our results demonstrated that hesperetin has therapeutic and preventive effects on the abnormal contraction induced by SPC. We observed the selective inhibitory effect of hesperetin on the SPC-induced abnormal contraction and its minor inhibitory effect on depolarization-induced contraction. We obtained slightly different results than those in a previous study by Liu *et al.* regarding the inhibitory effect of hesperetin on depolarization-induced vasoconstriction.[23] We speculated that the reason for the small difference might be related to the difference of animal species and the different potassium ion concentrations in our study. Although we aimed to investigate the direct effect of hesperetin on vascular smooth muscle, to some extent, the application of VSM tissues without the endothelium and adventitia may also be a limitation of our study. Additionally, we observed that the inhibitory effect of hesperetin was reversible when we replaced Krebs solution to wash out hesperetin after we observed a direct inhibitory effect of hesperetin on the SPC-induced contraction, suggesting that the target of hesperetin may be a receptor or transporter on the cell membrane. The specific target through which hesperetin inhibits the SPC-induced abnormal contraction is currently under investigation.

The pro-oncogene tyrosine-protein kinase Fyn is a member of the Src family of tyrosine kinases. Activation of Fyn is regulated by tyrosine phosphorylation and dephosphorylation of Fyn at two sites, Y420 and Y531 (in humans and pigs). While phosphorylation at Y420 in the activation loop of the kinase domain upregulates

enzyme activity, phosphorylation at Y531 in the carboxy-terminal tail by C-terminal Src kinase reduces the activity of the enzyme.[32] Recently, Fyn was shown to be involved in protein kinase IIδ2-mediated VSM cell motility.[33] The activation and translocation of Fyn play important roles in VSM contraction mediated by the SPC/ROK pathway.[8] In the present study, we demonstrated that hesperetin inhibits the SPC-induced abnormal VSM contraction and that hesperetin inhibits the SPC-induced translocation of Fyn from the cytoplasm to the cell membrane in HCASMCs and the activation of Fyn in both VSMs and HCASMCs. Although clinical studies on hesperetin are not presented in this paper, hesperetin is alike EPA, which is expected to inhibit vasospasm diseases based on the results that hesperetin suppresses the translocation and activation of Fyn.

Numerous studies have demonstrated that ROK plays an important role in Ca^{2+}-sensitization of VSM contraction leading to vasospasm. Phosphorylation of MYPT1 (T853) induced by ROK activation is correlated with vasoconstriction.[34] ROK binds directly to the myosin binding subunit of MLCP to regulate MLCP and MLC phosphorylation.[35] Additionally, ROK phosphorylates MLC20 at S19 to increase myosin ATPase activity, leading to cell contraction.[36] The principal mechanism of the regulation of smooth muscle contraction involves the phosphorylation and dephosphorylation of MLC20 at S19 that is regulated by the opposing activities of MLCK and MLCP.[37] MLCK phosphorylates MLC, leading to VSM contraction; however, MLCP dephosphorylates MLC, leading to VSM relaxation.[5] Our previous studies have confirmed that SPC induces the activation and translocation of ROK from

the cytoplasm to the cell membrane.[6, 7, 8] Activated ROK phosphorylates MYPT1 (T853), resulting in the inhibition of MLCP activity and leading to increased p-MLC (S19) and abnormal contraction of VSM.[35] The current results confirmed that hesperetin significantly inhibits the SPC-induced translocation of ROK from the cytoplasm to the cell membrane in HCASMCs, activation of ROK and phosphorylation of MLC in both VSMs and HCASMCs. Given these findings, we speculate that the mechanism by which hesperetin inhibits the SPC-induced abnormal contraction is closely related to indirect inhibition of the translocation and activation of Fyn and ROK via action on certain targets on the cell membrane, resulting in the inhibition of p-MYPT1 (T853) and p-MLC (S19) and consequently inhibiting VSM contraction. Presently, we are investigating the mechanism by which Fyn promotes ROK activation and are screening the candidate molecules that may link Fyn and ROK.

Analyses of the pharmacokinetic parameters of hesperetin have shown that the plasma concentration can reach up to approximately 80 μM in rats after the intragastric administration of 100 mg/kg of hesperetin and that the blood concentration in human volunteers after oral administration of 135 mg hesperetin can reach 10 μM.[38, 39] The hesperetin concentrations used in the present study were nearly in the same range as those *in vivo*. In addition to hesperetin, the main metabolite hesperetin 7-O-β-D-glucuronide in plasma has also been confirmed to induce vasorelaxation.[39] These findings provide basic support for the clinical development and utilization of hesperetin as a medicine to prevent and treat CVDs.

Conclusions

Our study demonstrated that hesperetin inhibits the SPC-induced abnormal contraction of VSMs. This inhibition occurs by suppressing the SPC-induced activation and translocation of Fyn and ROK from the cytoplasm to the membrane and subsequently inhibiting MLC phosphorylation (Fig. 6). The current study supports the idea that hesperetin benefits the treatment and prevention of abnormal VSM contraction caused by SPC and provides a theoretical basis and practical data for further exploration of hesperetin to treat and prevent CVDs.

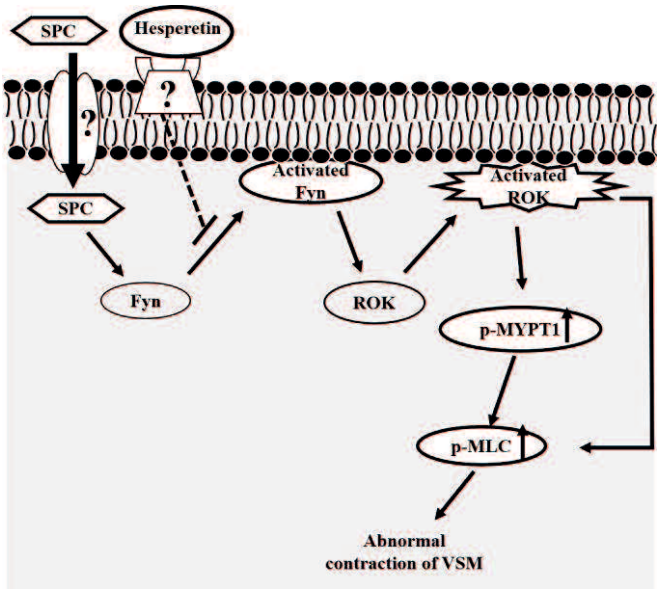

Figure 6. The proposed mechanism by which hesperetin inhibits the SPC-induced VSM contraction by inhibiting activation and translocation of Fyn and ROK. SPC stimulation causes abnormal contraction of VSM via the SPC/Fyn/ROK pathway. The SPC-induced translocation of Fyn and ROK from the cytosol to the cell membrane plays a vital role in the SPC-induced VSM contraction. In the present study, we demonstrated that hesperetin suppresses the SPC-induced VSM contraction via inhibiting the activation and translocation of Fyn and ROK and subsequent inhibition of MLC phosphorylation.

Acknowledgment

I have traveled a long way and suffered a lot before showing this doctoral dissertation to you. Four years of youth were fleeting, and past persons and events appeared before my eyes. It starts in the spring of 2018, and ends in the spring of 2022. At the city by the sea in Japan, I tasted all the bitter and sweet tastes of life.

In April 2018, I left my 28-month-old daughter in the care of my parents, and came alone to Yamaguchi University to study abroad for medicine doctor. At the beginning of 2020, the COVID-19 epidemic had swept the world, resulting in countries shut down. All kinds of pressures and anxiety from scientific research, paper publication, graduation, etc. brought me to the brink of despair. Those days seem to be the darkest time of my life. Fortunately, there are so many people love me, my family, friends, and teachers, who encouraged and comforted me to continue studying persistently. All the pain and frustration will create a new self.

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If home is a safe haven, the motherland must be a beacon for the overseas students to find the direction of home. Sincere gratitude to my motherland.

Turn our dream into a goal and motivation, and do not waste our youth. I finally like to express gratitude to myself for working hard consistently and persistently, and never giving up. I don't know what the future will be, fortunately, I can always find something in the process. Only love can offset the endless years. The romance of universe and the warmth in the world are worth my progress, and hard work for me means: when good luck comes, I am worthy.

The text has its strengths, while the gratitude is endless.

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